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Quinone Binding Sites of Membrane Proteins as Targets for Inhibitors*

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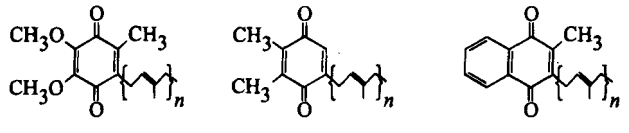
Abstract: Quinones are a central component of most photosynthetic and respiratory electron transfer chains. The proteins that reduce and oxidise these quinones have quinone binding sites—Q sites—that are good targets for pesticides. This paper reviews the diversity of these sites, their possible structure, and the types of compounds that act upon them.

Key words: biological electron transfer, Q sites, quinones, inhibitors, respiration, photosynthesis

1 INTRODUCTION

A common feature of most biological respiratory and photosynthetic electron-transfer chains is that they utilise at some stage the reduction and oxidation of quinones by redox proteins.[1] The three most commonly occurring quinones are shown in Fig. 1. Quinones are often one of the essential elements of the energy coupling process; by using the mobility of quinones between quinone-reactive sites (Q sites) arranged on proteins in a transmembrane manner, the coupling of electron transfer and proton translocation is achieved as part of a classical redox loop mechanism first described by Mitchell.[2]



Ubiquinone Plastoquinone Menaquinone

Fig. 1. The structures of biological quinones. Ubiquinone is a component of mitochondrial and many bacterial respiratory chains, whereas plastoquinone is found in higher plant and algal photosynthesis. Menaquinone can be found in place of, or in addition to, ubiquinone in bacterial systems. All forms are extremely hydrophobic because of the long poly-isoprenyl side chain: *n* is generally between 8 and 10 for ubiquinone and menaquinone and 9 for plastoquinone.

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Because of the central role of quinones in electron-transfer chains, many different Q sites are known. For many, rapid movement of the quinone on and off the site occurs and the binding constant is relatively weak. This dynamic association is required if the quinone is to act to couple electron and proton transfer, and it provides the possibility of inhibition of the transiently unoccupied site by a quinone analogue or other molecule. Other Q sites involve relatively immobile quinones, and displacement by an antagonist is more difficult. In this case the quinone remains bound during its redox cycle and acts simply as a component of the intra-protein electron-transfer chain.

Both synthetic and natural antagonists are known for a number of these sites in photosynthesis and respiration. All antagonists are relatively hydrophobic and considerable specificity for individual Q sites is often found. Differences in sensitivity of the same site in different species are known. Even a single amino acid change, which does not affect the catalytic activity of the enzyme, can cause a dramatic change in inhibitor sensitivity, and this has allowed the use of genetic methods to probe Q sites by analysis of inhibitor-resistant mutants.[3,4]

The purpose of this article is to review some of the wide range of Q sites that are already known and the types of inhibitor that act on them.

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2 THE STRUCTURE OF Q SITES

The binding sites of many cofactors or prosthetic groups often involve a characteristic protein fold whose primary sequence has a characteristic pattern for all such sites. The recognition of these protein sequence motifs has been of great value in protein structure prediction. To date, however, the high resolution structures of only two such Q sites in a membrane-bound protein are known, namely the Q_A and Q_B sites of the bacterial reaction centre[5,6] and these pockets are formed predominantly not by a single protein fold, but rather by a number of residues in the M (Q_A site) and L (Q_B site) subunits that are quite separated in their primary sequences. Hence, there is as yet no general sequence motif for a Q site for use as a tool to search for Q sites in proteins of unknown tertiary structure. Whether such a motif may become evident remains to be established when further Q site structures are elucidated.

Despite the lack of a clear motif, a number of common physical features of Q sites are likely. All such sites are relatively hydrophobic and are accessed by the quinone from the membrane phase, as necessitated by the hydrophobic nature of the quinone substrate. It seems likely that the predominant factor for binding into the site is the provision of groups at the correct distance so that hydrogen bonds can be formed with the quinone/quinol carbonyl/hydroxyl groups. For those sites (the majority) which interconvert the quinone, Q, and the protonated quinol, QH_2, access of the sites to an aqueous phase is required so that associated protonation reactions can occur. This could be direct or *via* a hydrophilic channel through the protein structure. In the case of the Q_B site, protonatable groups close to the quinone (GluL212 and AspL213), and polar routes of contact with the aqueous surface, can be recognised.[7] Lack of such access to protons radically changes the properties of the quinone in the site, such that it generally can only cycle between the oxidised, Q, and semiquinone, $Q^{\cdot -}$, forms.

A comparison of the structures around the Q_A and Q_B pockets[8] reveals several similarities (Q_A and Q_B residues given in brackets):

(i) a histidine group (HisM219 and HisL190) which is ligated to the common iron atom and which is likely to hydrogen bond to one of the quinone carbonyl groups. At least in the Q_A site, further hydrogen bonding of this carbonyl to the backbone nitrogen of AlaM260 (GlyL225 in Q_B) occurs;

(ii) hydrogen bonds between the protein and the second quinone carbonyl group. Originally, these hydrogen bonds were thought to arise from the uncharged polar groups, ThrM222 and SerL223. However, a more recent refinement of the structure of the Q_B site indicates that hydrogen bonding may instead be to the backbone peptide nitrogens of IleL224 and GlyL225;[9]

(iii) an aromatic residue (TrpM252 and PheL216) which is particularly close to the quinone ring in the Q_A site, but further away in the Q_B sites. An aliphatic residue (ValM226 and IleL229) occupies the opposite side of the quinone ring.[10]

The majority of the differences between the physical properties of quinone when bound in the Q_A and Q_B sites are likely to arise from the greater hydrophobicity, the more restricted access for quinone exchange, and the lack of protonatable groups of the Q_A site.

Since the M and L subunits are evolutionarily related, it is impossible at this stage to speculate with confidence on how many of the common features will be shared by other Q sites. However, it seems likely that some new structures which contain Q sites will be elucidated in the near future, and this may allow identification of a motif for recognition of other quinone binding sites. Recently, the crystal structures of mammalian[11] and bacterial forms of a soluble NAD(P)H quinone oxidoreductase have been determined. The structure of the mammalian enzyme with bound duroquinone has also been elucidated and this provides a third Q site structure, albeit of a soluble enzyme. Interestingly, this structure has features in common with the Q_A and Q_B sites in terms of hydrogen bonding (in this case of one quinone carbonyl to two tyrosine residues), interaction of the quinone ring with a coplanar ring structure (in this case the isoalloxazine ring of the flavin), and inhibition by Q site inhibitors.[12]

3 TYPES OF INHIBITOR

Inhibitors of Q sites are extremely diverse. Many are relatively simple (Fig. 2), with a clear resemblance to the structure of the natural quinones in terms of positions of hydrogen bonding groups and hydrophobic character. Some are redox inactive, at least in their mode of action (although unrelated electrochemical activity at extreme potentials is not uncommon). Such molecules have been discovered from natural, e.g. aurachins,[13] piericidin A[14] and synthetic, e.g. triazines and DCMU (3-(3′,4′-dichlorophenyl)-1,1′-dimethylurea), sources. It seems almost inevitable that these compounds bind in place of the quinone in the Q site. In some instances, structured water molecules may also be required to form the stable, inhibitory complex, as has shown to be the case for the binding of atrazine (a triazine) in the Q_B pocket of the bacterial reaction centre.[15]

Some of these simple molecules are themselves quinones or other redox active compounds. Although they presumably bind to the Q site in the same manner as substrate quinone, their physical properties are such that catalytic turnover cannot be completed. Many synthetic quinones are known in this category e.g. DBMIB (2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone) and various hydroxy-naphthoquinones. In some instances, a partial redox reaction can still occur. For

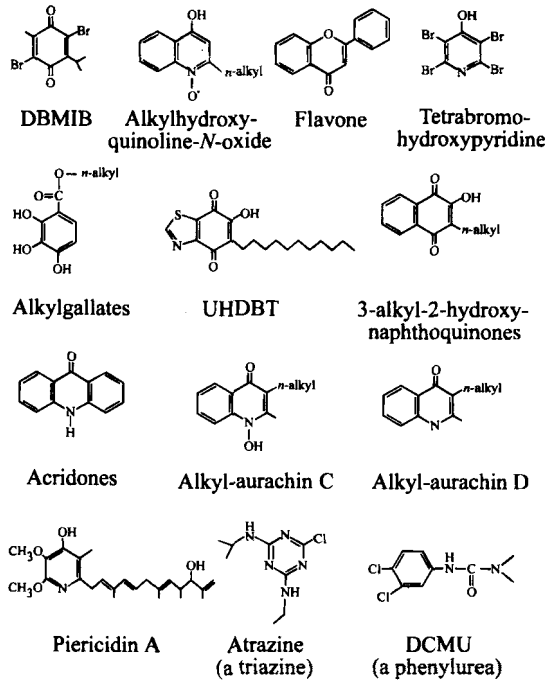


Fig. 2. Structures of some simple Q site inhibitors. Further information on these and related derivatives can be found as follows: DBMIB,[89] alkyl-HQNO;[79,90] flavones;[68] hydroxypyridines;[89] alkylgallates;[33,34] UHDBT;[91] alkyl-2-hydroxynaphthoquinones;[39] acridones;[64,92] aurachins;[13,93] piericidin A;[14] triazines and phenylureas.[37,89]

example, DBMIB is usually converted to its reduced state, $DBMIBH_2$, when added to a biological system such as chloroplasts, and is not an inhibitor of the Q sites of chloroplast cytochrome *bf* complex in this form. However, oxidation at one of the Q sites converts it into a bound form which is an extremely potent inhibitor.[16] In other instances, redox active chemicals may not bind strongly to a Q site at all, but show biological toxicity because they are biochemically reduced at the site and then autoxidise to produce toxic species such as superoxide anions.

Other Q site inhibitors have a more complex structure, and regions capable of binding in place of the substrate quinone are not so clear. Although direct binding to the Q site may still occur, significant interaction with the protein structure surrounding the Q site seems likely. Inhibition again probably occurs by blocking the binding of the natural quinone, or possibly its access route, to the site, although in some instances a more allosteric effect on the Q site has been postulated. The more complex molecules have been discovered initially from natural sources, e.g. antimycin, myxothiazol, rotenone and acetogenins such as bullatacin (Fig. 3 and Table 1). For two cases that have been tested,[9,17] the detailed molecular stereochemistry (Fig. 4) has been shown to be critical for activity. However, the full complexity of some natural compounds is not always essential. In the case of stigmatellin (Fig. 3), for example, the complicated tail can be replaced by a simple *n*-alkyl one of appropriate partition coefficient, and the crystal

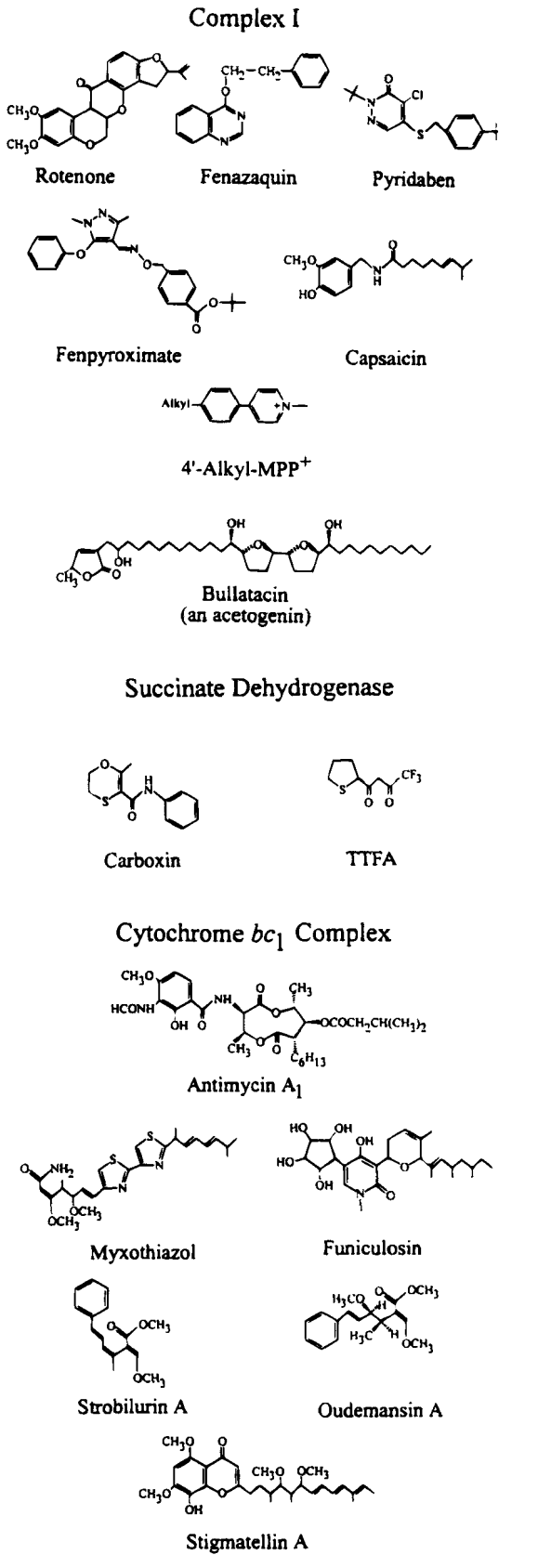
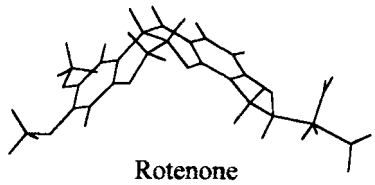


Fig. 3. Structures of some mitochondrial Q site inhibitors. References to further information on these compounds are given in Table 1.

TABLE 1
Q Sites and Inhibitors in Mitochondria

Enzyme	*Likely number of Q sites*	*Examples of tight,[a] relatively specific[b] inhibitors*	*Other inhibitors or relevant literature*
Complex I[c]	One or two[56,57]	Rotenone[14] Piericidin A[14] Acetogenins[58,59] Fenazaquin[27] Capsaicin derivatives[60]	MPP^+ [61–63] Others.[9,26,27,57,64]
NDH-2[65]	One	None	Refs 65, 66
$NADH_{ext}$ dehydrogenase[67]	One	None	Flavones[68]
Succinate dehydrogenase	Two[69]	Carboxins[70–72]	TTFA[71]
Electron-transferring flavoprotein[73,74]	One	None	
D-glycerol-3-phosphate dehydrogenase[74]	One	None	
bc_1 complex	Two[35] or three[75]	Antimycin A[76] Myxothiazol[77] Methoxyacrylate derivatives including oudemansins and strobilurins[78] Funiculosin[79] Stigmatellins[80]	Refs 81, 82
Ubiquinol oxidase[83]	At least one	Alkyl gallates[34]	Refs 30–32

[a] Tight binding is arbitrarily defined as an observed dissociation constant of 10^{-6} M or less in at least one species. However, dramatic variation between species can occur and such dissociation constants are in any case dependent on assay conditions and type of enzyme preparation.
[b] Showing a reasonable specificity for one Q site within the mitochondrial electron transfer chain, although cross-reactivity with Q sites in other electron-transfer systems occurs in some cases.
[c] NADH dehydrogenases are categorised as complex I (or NDH-1), NDH-2 (by analogy with categorisation of a second bacterial enzyme[65] and a third enzyme, $NADH_{ext}$ dehydrogenase, found in plants and fungi which oxidises cytosolic NADH and presumed to be a distinct enzyme type.


Rotenone

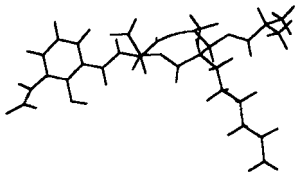

(+)-Antimycin A_1

Fig. 4. Conformations of some complex Q site inhibitors. The structures of two natural Q site inhibitors, rotenone and (+)-antimycin A_1, have been computed using the HYPERCHEM energy minimisation software. The calculated rotenone structure agrees well with that determined crystallographically.[94]

structure of a reaction centre-stigmatellin complex clearly shows that only the headgroup has specific interactions with the protein, to which it binds in a quinone-like manner.[15]

Regardless of complexity of structure, some inhibitors are tight and immobile, staying attached to a single site for many seconds, whereas others are tight but rapidly mobile on a millisecond timescale between identical sites in the same membrane.[18] The degree of inhibitor mobility is not directly correlated with quinone exchangeability in the site, and the factors which control the rate constants of inhibitor exchange are not understood.

4 SOME Q SITES AND THEIR INHIBITORS

4.1 All mitochondrial respiratory chains

The majority of mitochondrial respiratory chains possess a common basic set of components, and these

have perhaps been most studied in the mammalian mitochondrion. The pool of ubiquinone connects dehydrogenases such as NADH dehydrogenase (complex I) and succinate dehydrogenase (complex II) to the cytochrome bc_1 complex (complex III) and all three of these enzymes and their Q site reactivities have been studied in detail. Although a number of powerful inhibitors are already known for the majority of their known Q sites (Table 1 and Fig. 3), specificity for the enzyme in particular organisms, e.g. for fungal or insect forms of the enzymes, remains a possibility. For example, specificity of benzimidazole-*N*-sulfonamides for the bc_1 complex of *Pythium aphanidermtum* has been found.[19] In some instances, insensitivity of a species to a compound which inhibits the same enzyme in closely related organisms has been shown, e.g. an insensitivity to the *bc* complex in sea urchins to the classical inhibitors myxothiazol and strobilurin[20] or in fish to funiculosin.[21] Commercial applications of some compounds that act on these Q sites have been described.[22–27]

4.2 Plant, yeast and fungal mitochondria

Mitochondria from yeasts, fungi and higher plants can contain additional enzymes that are not found in mammalian systems (Fig. 5). In particular, a very active externally facing, inner membrane NADH dehydrogenase is often present. In addition, a second NADH dehydrogenase (termed NDH-2 in order to distinguish it from complex I) for oxidation of internally generated NADH is present which is insensitive to complex I inhibitors.[28] Although a variety of rather weak Q site inhibitors are known for these additional NADH dehydrogenases, no tight and specific inhibitors are yet available.

In addition to the extra dehydrogenases, mitochondria from these sources often possess a second 'alternative' oxidase which oxidises ubiquinol directly.[29] A variety of rather weak or non-specific inhibitors have been known for some time.[30] One of those most commonly used at present is *n*-propyl gallate, although concentrations in excess of 10^{-5} M are required for maximum inhibition.[31] Substitution of the *n*-propyl group for an octyl or dodecyl group increased the potency of inhibition of an analogous trypanosomal enzyme[32,33] and we found recently that the same change increased substantially the potency of inhibition of the plant enzyme, so that the inhibitor became stoichiometric in some conditions.[34]

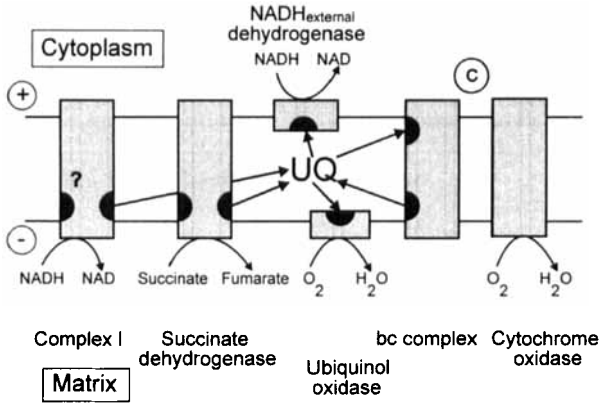


Fig. 5. Q sites of proteins of mitochondrial respiratory chains. Q sites are indicated by shaded semicircles. Arrows indicate normal direction of electron flow. A question mark indicates uncertainty on the existence of the site. Components are not universally present in mitochondria from different species. UQ = ubiquinone; c = cytochrome *c*.

4.3 Higher plant and algal chloroplasts

The higher plant photosynthetic electron-transfer chain consists of two photosystems, connected in series by plastoquinone, a cytochrome *bf* complex (homologous to the mitochondrial bc_1 complex) and plastocyanin. More than ten Q sites have been proposed,[35] but not all have withstood the test of time. Six of them are now well-documented (Fig. 6). However, a number of other functions recently recognised in thylakoids imply that other novel Q sites are likely to be found in the future.

Two of the sites are on photosystem II and are functionally similar to the Q_A and Q_B sites in the bacterial reaction centre. Indeed, the sequence homology between photosystem II and the bacterial reaction centre also suggests that they are similar, and structural models have been proposed on this basis, see Ref. 36. Because of its inaccessibility and the lack of exchangeability of the plastoquinone when bound, the Q_A site is not a good target for inhibitors. In contrast, a wide range of inhibitors can bind to the Q_B site and prevent reaction with plastoquinone.[36–40] Indeed, many commercially important herbicides, such as the triazines and phenylureas (Fig. 2), act on this site.

Two further Q sites, termed the Q_o and the Q_i sites, occur on the cytochrome *bf* complex. This enzyme is homologous to the mitochondrial and bacterial *bc* complexes and it is likely that the structures and function of the two Q sites are similar. However, the majority of the

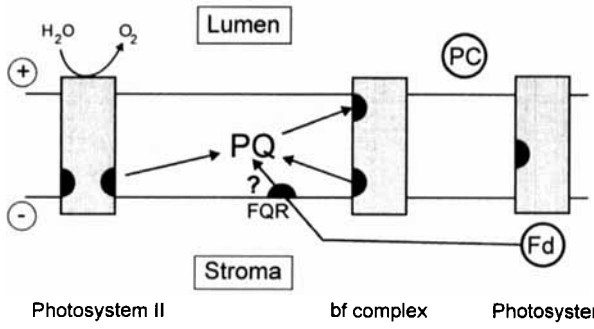


Fig. 6. Q sites in higher plant thylakoids. Conventions are as for Fig. 5. PQ = plastoquinone; PC = plastocyanin; Fd = ferredoxin; FQR = a putative ferredoxin-plastoquinone oxidoreductase.

very tight, specific, inhibitors of the Q sites of the *bc* complexes do not act on the chloroplast enzyme. Of the 'classical' mitochondrial Q_o site inhibitors, only stigmatellin (Fig. 4) acts in the same way on the cytochrome *bf* complex[1] and recently we have found that its potency can be improved by exchange of the natural sidechain for a simpler C_{13} *n*-alkyl one.[41] None of the classical *bc* complex inhibitors acts well on the Q_i site of the cytochrome *bf* complex. The first compounds shown to act on this site were HQNO and NQHO (2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide and 2-*n*-nonyl-4-hydroxyquinoline-*N*-oxide),[42] and we found improved specificity with MOA-stilbene (*E*-β-methoxyacrylate-stilbene or (*E,E*)-methyl 3-methoxy-2-(styrylphenyl)propenoate)[43] and, more recently, with a compound that we have termed FIQ (5-(3′,4′-dimethoxybenzyl)-2-(1′-methyl-2′-phenylthioethyl)-2,3,5,6,7,8-hexahydrofuro[2,3-*g*]isoquinoline).[41] However, we still have not achieved the potency and specificity seen with corresponding inhibitors of the Q_i site of the *bc* complexes. This, in combination with a number of other differences shown by the *bf* complexes, has even led some workers to consider that the chloroplast enzyme might work in a quite different way to the *bc* complexes, although we have argued that this view is not yet justified.[44] In any case, there is still a need to identify more powerful inhibitors of this site.

A further Q site appears within the photosystem I complex. The quinone, termed A_1, is a naphthoquinone, presumably tightly bound in a rather aprotic and apolar environment, and acting as a simple electron-transfer component between chlorophyll and the iron sulphur centres by cycling between its oxidised and semiquinone forms.[45] Although such a site might be presumed to be difficult to inhibit competitively, such studies have been reported,[46,47] and this site remains a novel target.

It is known that photosynthetic ATP production can occur by electron transfer around photosystem I without the involvement of photosystem II. This process of cyclic photophosphorylation may be the only one that occurs in some types of plant cell[48] but the degree to which it occurs in more conventional thylakoids remains uncertain. It is known to require ferredoxin, which accepts an electron from photosystem I and passes it back to plastoquinone *via* a ferredoxin-plastoquinone oxidoreductase termed FQR.[49] Although this FQR protein has not yet been characterized, it does appear to have a unique Q site which interacts with plastoquinone and it is this site which is likely to account for the sensitivity of the process to antimycin A and other likely Q site inhibitors.[50,51] Again, a more thorough investigation of other possible inhibitors of this Q site is warranted.

Besides these sites, there are a number of more enigmatic Q sites that are likely to be operative in the more intact *in-vivo* situation. For example, a number of additional sites in intact cells may be present which control a slow reduction and reoxidation of the plastoquinone pool which can occur in the dark in response to other physiological changes of the cell. These processes have collectively been termed 'chlororespiration' and have been suggested to arise from specific thylakoid forms of NADH dehydrogenase and plastoquinol oxidase.[52] However, direct evidence for such enzymes in higher plant or algal thylakoids is poor and the molecular nature of dark plastoquinone reactions has yet to be established. A further example is provided by a major medium-term control mechanism of protein phosphorylation/dephosphorylation, which controls the balance of photosystem activities and responds to the redox state of the plastoquinone pool.[53] This implies that the proteins involved in sensing redox state changes are likely to have sites specific for the Q or QH_2 forms of plastoquinone. The identification of specific inhibitors for any of these sites would provide a major tool for understanding their molecular origin and might in turn provide new target sites for herbicides.

4.4 Bacterial systems

Since the mitochondrial and thylakoid systems evolved from bacterial origins, it is not surprising that homologues of the majority of enzymes described above can be found in the electron-transfer chains of various bacterial species. Generally, inhibitors that act on the eukaryotic enzymes are also effective on their bacterial counterparts. In addition, however, there is a range of more specialised enzymes in different bacteria for the reduction or oxidation of the quinone by diverse metabolic substrates, and more are likely to be discovered. Table 2 summarises some of these bacterial enzymes. Even for a particular type of enzyme, great diversity of

TABLE 2
Some Additional Bacterial Enzymes Which May Have Q Sites

Enzyme	*Donor or acceptor*	*References*
Quinone-Reducing[a]		
Hydrogenase	Hydrogen	74
NDH-2	NADH	65
Na^+-NQR	NADH	84
Formate dehydrogenase	Formate	85
Glucose dehydrogenase	Glucose	86
Quinol-Oxidising[a]		
Cytochrome *bo*	Oxygen	87
Cytochrome *bd*	Oxygen	87
Nitrate reductase	Nitrate	74
DMSO reductase	Dimethyl sulphoxide	74
TMAO reductase	Trimethylamine-*N*-oxide	74
Fumarate reductase	Fumarate	88

[a] The quinone system in bacteria may be ubiquinone, menaquinone or both.

detail is found between homologues in different bacterial species (e.g. the variants of the oxidase superfamily[54]). Recently, we screened a range of compounds as possible inhibitors of the Q sites of the two principal bacterial quinol oxidases, cytochromes *bo* and *bd*, and identified a number of compounds that are powerful inhibitors of these sites.[55] However, inhibitors of the Q sites of many of the other bacterial enzymes have yet to be identified.

5 CONCLUSIONS

The Q sites form a wide and diverse class of target sites for specific inhibitors and it has yet to be established whether they have a common primary sequence motif. Although they are generally rather simple and non-specific for the types of quinone that they can recognise, great specificity for individual sites is often exhibited by inhibitors. The search for new inhibitors offers exciting prospects both for aiding the elucidation of the molecular properties of the enzymes and for providing new types of commercially viable pesticides.

ACKNOWLEDGEMENTS

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